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Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts

Jeanette Hammer Andersen [a,*], Svein Are Osbakk [a], Lars Harry Vorland [a,c], Terje Traavik [b], Tore Jarl Gutteberg [a,c]

[a] *Department of Microbiology, University Hospital of Tromsø, N 9038 Tromsø, Norway*
[b] *Department of Virology, Institute of Medical Biology, School of Medicine, University of Tromsø, Tromsø, Norway*
[c] *Department of Medical Microbiology, Institute of Medical Biology, School of Medicine, University of Tromsø, Norway*

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Abstract

Lactoferrin is mainly produced by polymorphonuclear leukocytes and has been demonstrated in mammalian milk and external secretions. Lactoferrin is an iron-binding, multifunctional protein and may play an important role in immune regulation and in defense mechanisms against bacteria, fungi and viruses. Lactoferricin is a potent antimicrobial peptide generated from the N-terminal part of lactoferrin by pepsin cleavage. We demonstrate that lactoferrins from different species and its N-terminal peptide lactoferricin (particularly the cyclic form) inhibit expression of early and late antigens, as well as production of infectious viral progeny during human cytomegalovirus (HCMV) infection in vitro. Iron-saturated lactoferrin did not affect HCMV antigen expression. Heparin had the same effects as iron-depleted lactoferrin. Yet, mixtures of lactoferrin and heparin did not inhibit HCMV multiplication i.e. lactoferrin and heparin seemed to mutually block each other's antiviral activities. HCMV-infected cells exposed to lactoferrin and cyclic lactoferricin contained less intracellular virus than unexposed cells. The antiviral activity of cyclic lactoferricin was more than seven-fold weaker than that of the maternal molecule. Lactoferrin and cyclic lactoferricin prevented HCMV entrance into the host cell.

Keywords: Human cytomegalovirus (HCMV); Lactoferrin; Lactoferricin

1. Introduction

Human cytomegalovirus (HCMV), classified within the *Herpesviridae* (Mocarski, 1995), may cause diseases like mononucleosis syndrome and hepatitis in the immunocompetent host. Between 0.2 and 2.2% of all newborn children are congenitally infected, and about 15% of these show clinical symptoms from the nervous and other organ systems. Furthermore, HCMV infection may complicate allograft transplantation and may contribute to progression of HIV infection to AIDS (Britt and Alford, 1995). Hence, preventive or therapeutical intervention in HCMV infections is important.

* Corresponding author. Tel.: +47-776-27043; fax: +47-776-27015.
E-mail address: mlabjeh@rito.no (J.H. Andersen).

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Lactoferrin (LF) is a protein found in milk, on mucosal surfaces and in polymorphonuclear leukocytes (PMN) (Masson et al., 1966). LF is a multifunctional protein of approximately 80 kDa and a part of the innate immune defense (Shau et al., 1992). Stimulation of PMNs leads to the release of LF, and, hence, LF is an early indicator of inflammatory state (Gutteberg et al., 1984, 1988). The molecular structure of LF has been revealed (Anderson et al., 1989; Day et al. 1992, 1993), and LFs from human, goat, mouse, cow, swine and sheep have been characterized (Metz-Boutigue et al., 1984; Le Provost et al., 1994; Cunningham et al., 1992; Pierce et al., 1991; Lydon et al., 1992; Qian et al., 1995). The N-terminal and C-terminal sequences of LF are symmetrical and each bind one Fe^{+++}.

Heparin and heparan sulfate belong to a family of cell-membrane inserted glycosaminoglycans, and heparan sulfate participates in the attachment and internalization of HCMV (Neyts et al., 1992; Kari and Gehrz, 1992; Compton et al., 1993). LF is known to bind to heparan sulfate proteoglycans and to the low-density-lipoprotein receptor (Ji and Mahley, 1994). The first 33 amino acid residues in the N-terminal part of LF engages in binding to glycosaminoglycans like heparin and heparan sulfate (Zou et al., 1992; Ji and Mahley, 1994; Mann et al., 1994).

Lactoferricin is generated upon gastric cleavage of LF (Tomita et al., 1991) and corresponds to the N-terminal part of LF. Lactoferricin makes a cationic distorted β-sheet joined together by a disulfide bridge (Hwang et al., 1998). In LF, the peptide structure forms a loop with a cationic charge at the tip.

Lactoferricin derived from the bovine LF is active against a wide range of Gram-negative and Gram-positive bacteria, fungi, protozoa and tumors (Bellamy et al., 1992, 1993; Isamida et al., 1998; Turchany et al., 1995; Yoo et al., 1997). Bovine lactoferricin (lactoferricin B) consists of 25 amino acid residues (17–41 in bovine LF) and human lactoferricin (lactoferricin H) consist of 47 amino acid residues including a region homologous to lactoferricin B.

Earlier studies have established that LF exerts antiviral activity against HCMV, human immunodeficiency virus (HIV), herpes simplex virus (HSV) and hepatitis C virus (HCV) (Harmsen et al., 1995; Hasegawa et al., 1994; Fujihara and Hayashi, 1995; Ikeda et al., 1998). It has been suggested that LF inhibits an early step in viral infection (Hasegawa et al., 1994).

The aim of this study was to elucidate if LF and lactoferricin could prevent HCMV entry into human fibroblasts either by binding to the virion or by blocking host cell receptors.

In the present report, we describe the inhibitory effects of LFs and lactoferricin on HCMV infection in cell culture.

2. Materials and methods

2.1. Reagents

Heparin (200 IE per mg) was purchased from LEO, Denmark. Human lactoferrin (55839) was a gift from Cappel/Organon Teknika Corporation, Durham, NC. The following items were purchased from Sigma Co, St. Louis, MO, human lactoferrin, human iron-saturated lactoferrin, bovine lactoferrin. Goat lactoferrin was purified from goat milk (gift from Tine Dairy, Tromsø, Norway) as earlier described (Gutteberg et al., 1984). Cyclic lactoferricin B was a gift from Morinaga Milk Industry, Zama City, Japan. Linear lactoferricin B, linear and cyclic lactoferricin H, and linear lactoferricin G (goat) were synthesized as described elsewhere (Vorland et al., 1998). The linear and cyclic human lactoferricin used in this study, consisted of amino acid residues 18–42 in human LF with a molecular weight of 3005 Da.

Briefly, the peptides (linear lactoferricin B, linear and cyclic lactoferricin H, and linear lactoferricin G) were solid phase synthesized by fluorenylmethoxycarboxyl chemistry, employing a 9050 Plus Pep Synthesizer (Millipore Corporation, Milford, MA). The synthesis was surveyed by continuous spectrophotometry. The cyclic peptides were provided with protective groups for cysteines. These were then split off and optimized to create an S–S bridge. Peptides were purified on a Waters 600 E HPLC (Millipore Corporation, Milford), and eluted fractions were analyzed by

liquid spectrometry-mass spectrometry (LC-MS) with electro-spray interface (G Quattro LC MS) and/or fast atom bombardment mass spectrometry (FAB-MS, Fisons VG Tribid). Purified peptides were stored lyophilized until use. All lactoferrins and lactoferricins were dissolved in distilled, pyrogen-free water to make stock solutions that were added to cell growth media to reach the final working concentrations reported below.

2.2. Virus and cell cultures

Stocks of HCMV AD169 (human cytomegalovirus strain AD 169, ATCC VR-538) were produced by propagation in MRC-5 cultures. MRC-5 cells (human diploid lung fibroblasts, ATCC CCL 171) were grown in minimum essential medium (Kari and Gehrz, 1992) with Ultroser G, Hepes buffer, non-essential aminoacids (Gibco, Paisley, Scotland), and regularly checked for Mycoplasma contamination (Ridascreen, r-biopharm, Darmstadt, Germany). MRC-5 cells were used as monolayers in 50-ml culture flasks, 96-well culture plates or 24-well culture plates.

2.3. Experimental design

HCMV infections were initiated by application of properly diluted stock virus to MRC-5 monolayers followed by centrifugation of the cell culture trays at $700 \times g$ for 40 min. The HCMV dilutions and the test substances: lactoferrins, lactoferricins or heparin, were added to cell cultures simultaneously. After a virus adsorption period of 4 h, the inoculums were removed and, in some experiments, applied to a new set of fibroblasts to detect infectious HCMV in the supernatant. Deviations from this scheme are given in the text.

2.4. HCMV infectivity assays

Infectivity assays were based on immunocytochemical detection of HCMV immediate early antigen (43 kDa protein) by monoclonal antibody DDG9, and delayed early protein (76 kDa protein) by monoclonal antibody CCH2. The antibodies were used according to the instructions of the Manufacturer (DAKO, Denmark). Late HCMV antigen (LA) expression was monitored by monoclonal antibodies (DAKO, SYVA Mikro Trak, Palo Alto, CA, Chemicon Temecula, CA).

Briefly, 16–18 h after HCMV infection and treatment with lactoferrins, lactoferricins and heparin, test and control cultures were fixed and stained according to an immunofluorescence or-peroxidase protocol (Flaegstad and Traavik, 1987). At the chosen time-point post infection (p.i.), there was neither newly produced virus nor secondary infection of MRC-5 cells. Accordingly, the number of cells expressing viral antigen(s) directly reflect the number of infectious virions in the viral inoculum. This method, applied to the human polymavirus (BKV) has been published elsewhere (Moens et al., 1994). This approach was used to quantify HCMV infectivity in stock virus preparations, and to determine the infectious doses during test substance treatment. Spectrophotometric readings were performed at 492 nm for peroxidase enzyme reactions. In preliminary experiments, it was demonstrated that the OD values obtained could be correlated with the counted number of HCMV-antigen expressing cells.

The calculations of IC_{50} values were based on the Median effect principle of Chou and Talalay (Chou and Talalay, 1984). This method involves the plotting of dose-effect curves for each agent and calculation of the IC_{50} value.

2.5. Immunofluorescence analysis

Coverslips were included when cells were seeded in 24-well culture plates. At 2 h p.i., all coverslips were simultaneously fixed and permeabilized by incubation for 10 min with 100% methanol at -20°C. Coverslips were blocked with normal goat sera (Jackson ImmunoResearch), then incubated with mAb to pp65 (Goodwin Institute, Plantation, FL). Primary Ab was detected with goat anti-mouse IgG (Jackson ImmunoResearch) coupled to fluorescein. All samples were counterstained with Evans blue to visualize the cells. Slides were analyzed with a Leica DMR microscope fitted with a Leica DC 100 digital camera to capture images.

3. Results

Fig. 1 summarizes data from experiments comparing the relative HCMV inhibitory effect of lactoferrins from different species. It also compares the effects of the iron-saturated and apo-forms of human lactoferrin. The bovine version was the most effective, reducing the HCMV antigen expression by 90–100% at concentrations of 250–500 μg/ml (IC_{50} ~55 μg/ml (0.7 μM)). The apo-form of human lactoferrin was slightly, and the goat version markedly, less inhibitory than the bovine, exhibiting IC_{50} values of 91 μg/ml (1.1 μM) and 275 μg/ml (3.4 μM), respectively. The iron-saturated version had no inhibitory effect at the concentrations tested.

The antiviral effect of LF might, in toto or in part, be exerted by the domain corresponding to lactoferricin. Also, the addition of cyclic lactoferricin B during the HCMV cell-attachment period resulted in inhibition of viral IE/E antigen expression. The number of antigen-expressing cells was reduced to 50% by 15 μg lactoferricin per ml (5 μM), while 100 μg/ml (33 μM) left only an occasional IE/E antigen-expressing cell.

The relative effects of linear and cyclic lactoferricins were compared. As shown in Fig. 2, the cyclic human lactoferricin had a pronounced inhibitory effect on HCMV IE/E antigen expression. At a concentration of 100 μg/ml (33 μM), virtually no HCMV infectivity was detected. The linear forms of human and bovine lactoferricin had no antiviral effect, while some antiviral effect was seen with linear goat lactoferricin.

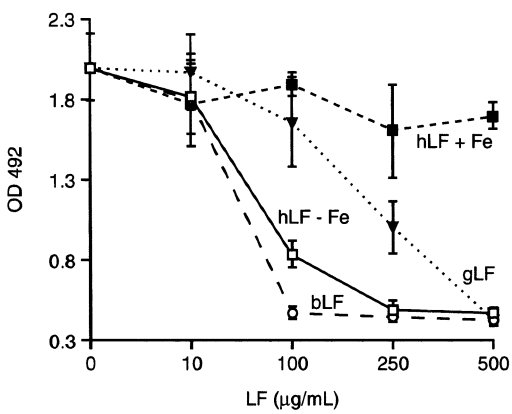

Fig. 1. Antiviral effect of lactoferrin from different species. Bovine lactoferrin (bLF), goat lactoferrin (gLF) and human lactoferrin in its apo-form (hLF − Fe) and iron-saturated form (hLF + Fe) were tested. HCMV and lactoferrin were added simultaneously to the cell culture. The IE/E antigen expression is monitored by the optical density at 492 nm as a function of concentration (μg/ml). Bars indicate standard deviation (S.D.).

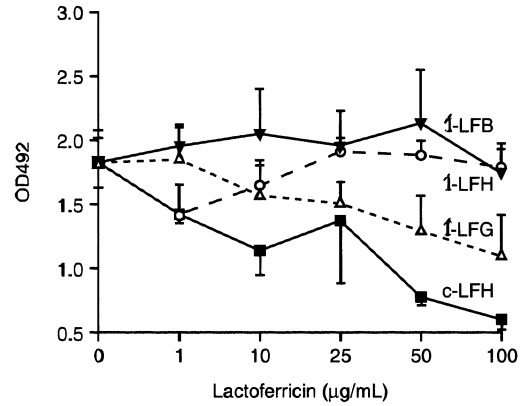

Fig. 2. Antiviral effects of different lactoferricins; human linear lactoferricin (l-LFH), human cyclic lactoferricin (c-LFH), goat linear lactoferricin (l-LFG) and bovine linear lactoferricin (l-LFB) are demonstrated. HCMV and lactoferricin were added simultaneously to the cell culture. IE/E antigen expression is monitored by the optical density at 492 nm as a function of concentration (μg/ml). Bars indicate standard deviation.

To identify the stage in the HCMV infection, where cyclic lactoferricin exerted its inhibiting effect, two parallel sets of experiments were set up. In one set of experiment, HCMV and lactoferricin were applied simultaneously to the MRC-5 monolayers. In the other set, equal parts of virus and lactoferricin were mixed and left at 37 or 0°C for 1 h before being added to the cell monolayers. Preincubation of the virus with lactoferricin did not result in greater inhibition. As shown in Fig. 3, the inhibitory effect on HCMV antigen expression was not affected by any of these preincubation procedures, thus indicating a mainly cellular target.

The effect of adding lactoferrin and lactoferricin to cell cultures at different time-points in relation to HCMV infection was investigated. In preliminary experiments, it was demonstrated that preincubation of the cell cultures for up to 2 h

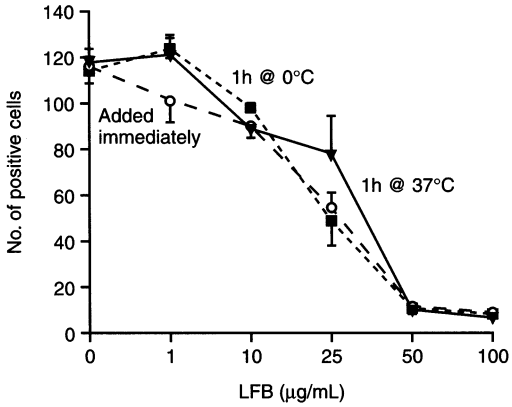

Fig. 3. The antiviral effect of cyclic lactoferricin B (LFB) when virus and peptide was preincubated at 0°C (■) and 37°C (▼) for 1 h before being added to the cell culture, compared with immediate addition of lactoferricin B and virus (○). IE/E antigen expression is indicated by the number of positive cells as a function of concentration (µg/ml). Bars indicate standard deviation.

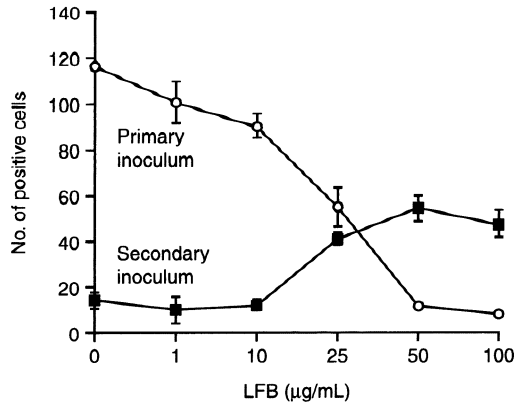

Fig. 5. Residual viral infectivity after earlier exposure to cyclic lactoferricin B (LFB). Primary inoculum contained HCMV and increasing concentrations of lactoferricin as given on the *X*-axis. Secondary inoculum is defined as the medium containing residual HCMV at the end of the virus adsorption period. Bars indicate standard deviation.

before the viral infection was initiated, did not enhance the inhibitory effect obtained by adding lactoferrin or lactoferricin and HCMV simultaneously.

If lactoferricin B was added 1 h after HCMV infection, the inhibitory effect on IE/E antigen expression was markedly reduced (Fig. 4). This indicated that the virus was not accessible to

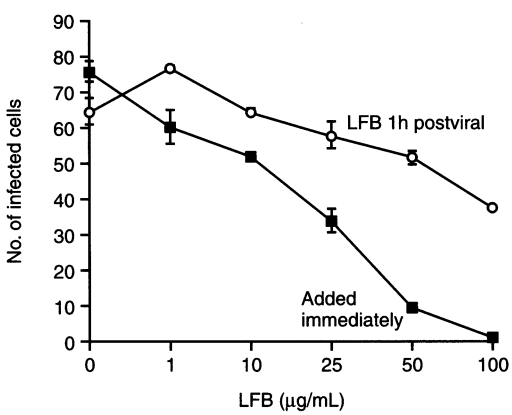

Fig. 4. Antiviral effect of cyclic lactoferricin B (LFB) added 1 h after virus infection compared with simultaneously addition of cyclic lactoferricin B and virus. IE/E antigen expression is indicated by the number of positive cells as a function of concentration (µg/ml). Bars indicate standard deviation.

treatment after it had entered the host cells. The inhibitory effect was also reflected in late antigen (LA) expression (data not shown).

Reduced antiviral effect when lactoferricin B was added after infection was also observed for human LF. When LF was added 2 h after HCMV infection, neither IE/E expression nor viral infectious progeny production was affected (data not shown).

Experiments were designed to reveal whether cyclic lactoferricin had a direct inhibitory effect on HCMV virions present in the inoculum, or acted indirectly by affecting the virus admission to host cells. The medium containing residual HCMV was harvested by the end of the virus adsorption period (2 h post infection), and transferred to new MRC-5 cultures for quantitation of residual HCMV infectivity. It became evident that increasing lactoferricin concentrations were accompanied by gradually higher residual yields of infectious HCMV in the inocula (Fig. 5). It appears that as lactoferricin concentrations increased, less virus was able to adsorb to cells and, therefore, remained in the medium. This indicated that lactoferricin does not primarily bind to, or affect the integrity of the virions.

Next, the net effect of having both heparin and lactoferrin present in our HCMV/fibroblast model

was examined. For this purpose, the apo-form of human lactoferrin and heparin in various concentrations, one at a time or mixed together, were present in the MRC-5 culture media during a 4 h HCMV attachment period. The results showed that at high heparin-concentrations (50 and 100 μg/ml), the inhibitory effect on HCMV was mainly due to heparin. This was irrespective of the presence of lactoferrin (Fig. 6). As the heparin-concentrations were reduced from 20 μg/ml to zero, the inhibitory effect of both compounds were abolished, probably by binding to each other. The net result was an increased number of HCMV-expressing cells. Finally, when the heparin-concentrations became low, the inhibitory effects of lactoferrin on HCMV were again evident. Titration of infectivity from the recovered cell culture media demonstrated the inverse situation — high remaining levels of HCMV virions from wells with low numbers of HCMV-expressing cells (data not shown).

Also the iron-saturated form of human lactoferrin and bovine lactoferricin were able to block the inhibitory effect of heparin on HCMV (data not shown).

Viral entry was affected by LF and lactoferricin as measured by a decreased number of pp65 tegument protein positive cells. (This is visualized by pp65 staining in the cell nuclei). As demonstrated in Fig. 7, treatment with 50 μg/ml (16 μM) lactoferricin B or 50 μg/ml (0.6 μM) bLF reduced the amount of pp65 positive cells. Concentrations ranging from 25 to 1000 μg/ml lactoferricin B or bLF were tested, and the inhibition of viral entry was dose-dependent. No positive cells could be detected after treatment with 1000 μg/ml of lactoferricin B or bLF.

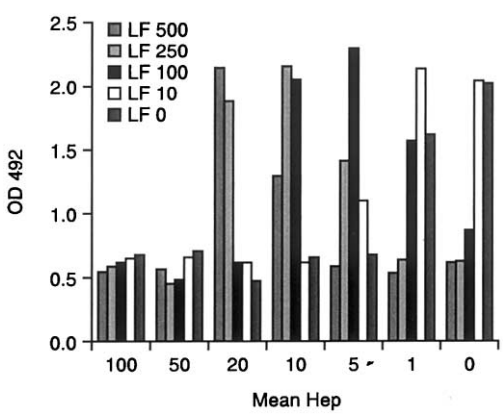

Fig. 6. The antiviral effect of heparin and LF in combination. IE/E antigen expression is monitored by the optical density at 492 nm as a function of heparin concentration in μg/ml. The columns represent the five concentrations of (in μg/ml) LF tested at each heparin concentration.

4. Discussion

In this report, the antiviral effects of LF and lactoferricin were investigated. We demonstrate that goat LF and the N-terminal peptide of different LFs, lactoferricin inhibit HCMV multiplication in vitro. Earlier, it has been well established that both human and bovine LF inhibit HCMV infection (Hasegawa et al., 1994; Swart et al., 1998. Hasegawa et al. (1994) suggested that the antiviral activity resulted from interference with an early event of virus infection. In this study, we have tried to identify at which stage in the viral infection, LF and cyclic lactoferricin exert their antiviral effects.

LF was on a molar basis more efficient than lactoferricin, indicating that the size of the molecule may be of importance, or that other regions of the LF molecule may contribute to the antiviral activity.

The difference in antiviral activity between bovine, human and goat LF may be due to some small structural/conformational changes between these molecules. Only the apo-form of human LF was able to inhibit HCMV infection in our study. Distinct conformational changes are induced by iron-binding, and these may alter the function of the molecule (Baker et al., 1994). Others have shown that both apo-LF and the iron-saturated LF of bovine origin have activity against herpes simplex virus and HCMV (Marchetti et al., 1998; Hasegawa et al., 1994).

The structure/conformation of the molecule may also be of importance for the antiviral activity of lactoferricin. The secondary structure of lactoferricin is stabilized by a disulfide bond formation. Lactoferricin must be in its cyclic form to exert antiviral activity. The absence of the

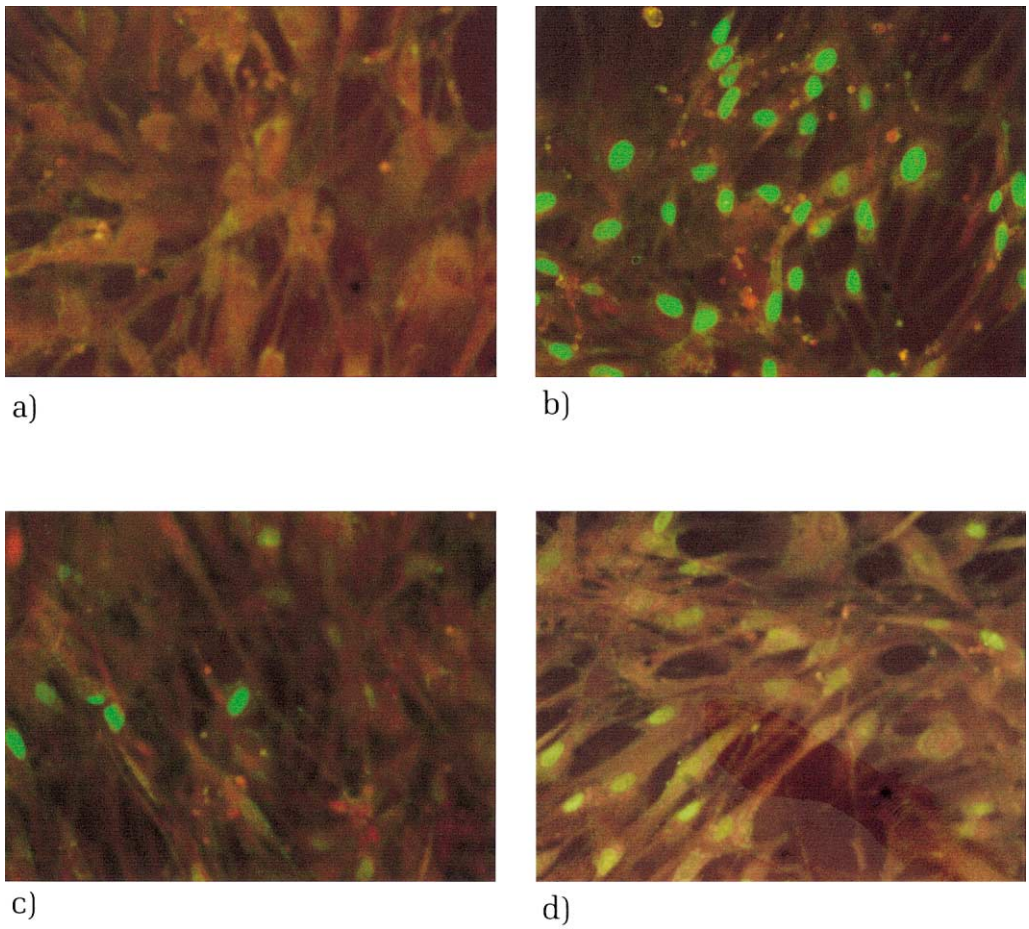

Fig. 7. The effect of LF and cyclic lactoferricin B on the expression of pp65 tegument protein (indicative of viral entry): (a) Uninfected cells; (b) HCMV-infected cells; (c) cells exposed to HCMV and 50 μg/ml cyclic lactoferricin B; (d) cells exposed to HCMV and 50 μg/ml lactoferrin.

disulfide binding in the linear peptide may be the cause of the loss in antiviral activity. Vorland et al. (1998) found that the antibacterial activity of lactoferricin B also decreased, when the cysteine disulfides were reduced.

It is possible that LF and cyclic lactoferricin exert their antiviral effects through different mechanisms; either by a direct effect on the virions or by an effect on the host cell. These mechanisms need not to be exclusive. We showed that neither LF nor cyclic lactoferricin B had any distinct effect directed against extracellular virions. Preincubating cyclic lactoferricin or LF and virus did not affect the inhibitory effect. After the virus adsorption period, a gradually higher level of residual HCMV infectivity was observed with increasing lactoferricin concentrations. Additionally, we observed no effect of LF or cyclic lactoferricin on viral protein production after the virus had entered the cell. This indicates that the antiviral effect is exerted primarily at the cell surface level.

The inocula used in our experiments consisted of harvested virus-containing media from HCMV-inoculated cell cultures. It might be speculated that bioactive molecules present in such prepara-

tions could contribute to or interfere with the observed lactoferricin-mediated antiviral activities. We did, however, show in control experiments that purified and unpurified HCMV batches from the same cell culture harvest had their IE/E antigen expression equally reduced by cyclic lactoferricin.

The initial event in HCMV infection is attachment to extracellular heparan sulfate. These molecules may act as host cell receptors for the virus (Compton et al., 1993). Earlier reports have demonstrated the ability of heparin and related compounds to inhibit HCMV infection (Compton et al., 1993; Kari and Gehrz, 1992; Neyts et al., 1992). The present study shows that the antiviral effects of heparin and lactoferrin were lost, when they were mixed together. This indicates that heparin and LF may interact with each other and hence exert no inhibitory activity on HCMV infection, when mixed together.

Antiviral agents that interfere with the interaction between the virus and host cell receptors may effectively block HCMV adsorption and entry. This blocking will affect the appearance of pp65, a tegument protein rapidly transported to the nucleus of infected cells (Schmolke et al., 1995). Viral entry was affected by treatment with cyclic lactoferricin and LF, as indicated by a decrease in pp65 positive cells. The ability of HCMV to enter the host cell was reduced, when increasing concentrations of either cyclic lactoferricin or LF were added.

Initiation of HCMV infection probably involves sequential interaction between several viral and cellular components, indicating that inhibition of this event in the viral infection could have multiple targets. The possibility of lactoferrin and cyclic lactoferricin to interfere with host cell components other than heparan sulfate can, therefore, not be excluded.

In conclusion, lactoferrin and cyclic lactoferricin inhibit HCMV infection of human lung fibroblasts in a dose-dependent manner. Lactoferrin and cyclic lactoferricin effectively block HCMV infection of human lung fibroblasts, possibly by affecting virus interactions with heparan sulfate on the cell surface.

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